Investigation of the Structure of Trifolirhizin, an Antifungal Compound from *Trifolium pratense* L.

J. B-SON BREDENBERG and
PENTTI K. HIETALA

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

In an investigation of the antifungal substances in red clover (*Trifolium pratense* L.) the isolation of one of the main components, $C_{21}H_{24}O_{10}$, m.p. 139–140°, has been reported [1]. This compound has now been investigated more closely. Since it is apparently a new compound, isolated so far only from the roots, the name trifolirhizin is proposed for it.

Trifolirhizin crystallizes from methanol as rods, m.p. 142–144° (decomp.), $[\alpha]_D^{20}$ −183°, with one mole of methanol. The elementary composition of the compound has now been found to agree with $C_{22}H_{22}O_{10}$. It does not contain methoxyl or C-methyl groups and, according to the chemical and infrared data, is free from carbonyl and carboxyl groups. Upon catalytic hydrogenation it is recovered unchanged. It forms a tetraacetate, $C_{30}H_{30}O_{14}$, m.p. 188–189,° $[\alpha]_D^{20}$ −126° in which all the hydroxyl groups are acetylated. Upon acid hydrolysis it forms glucose and ether-soluble substances which could not be characterized. Alkaline hydrolysis probably also releases glucose. Trifolirhizin is thus a glucoside.

Taking the glucose molecule into account, the empirical formula for the aglycone of trifo irhizin is $C_{16}H_{12}O_5$. One of the oxygen atoms is found in the glucoside bond. Since trifolirhizin contains four hydroxyl groups, all belonging to the glucose part, and since it does not have any carbonyl or carboxyl groups, the remaining four oxygen atoms in the aglycone must be of ether type. The infrared spectrum supports the presen e of a methylenedioxy group [2]. The presence of glucose, however, makes the normal colour test [3] for this group of little value.

The low hydrogen content and resistance to hydrogenation can only be explained if two benzene rings are assumed to be present. The very peculiar ultraviolet spectrum (Fig. 1) can be interpreted according to this assumption: the maxima at 280 and 285 mμ are formed from a slightly substituted benzene ring and the maximum at 310 mμ from a strongly substituted benzene ring. The latter maximum, however, occurs at a surprisingly high wavelength.

Taking into account the occurrence of *iso*flavones in clover [4-6] and their antifungal properties [7-9] a compound of pterocarpin (I) [10] type could be assumed to be present. When the ultraviolet and infrared spectra of a sample of pterocarpin, kindly supplied by Dr. W. B. Whalley, of Liverpool, were compared with those of trifolirhizin, a striking similarity was observed (Figs. 1 and 2). The ultraviolet spectra show that the compounds have identical chromophores. Since pterocarpin is methylated at the 7-position and trifolirhizin does not contain any methoxyl group, the glucose is evidently bound to the aglycone at this point. This would also explain [11] the suspected alkali lability of trifolirhizin. On the basis of the above results the structure II is therefore tentatively suggested for trifolirhizin.

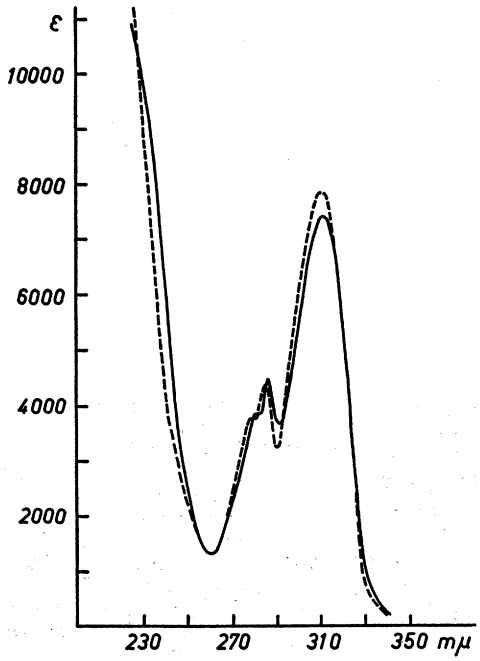
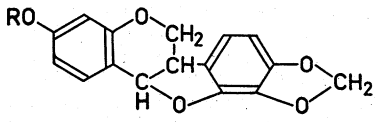


Fig. 1. Ultraviolet spectra in ethanol of pterocarpin (solid line) and trifolirhizin (broken line).



I: R=CH_3
II: R=Glucose

It is of interest to note the recent report [12,13] of the isolation of an antifungal compound from *Pisum sativum* L. which seems to bear a close relationship to the above-mentioned compounds. Lack of details, however, precludes further comparison.

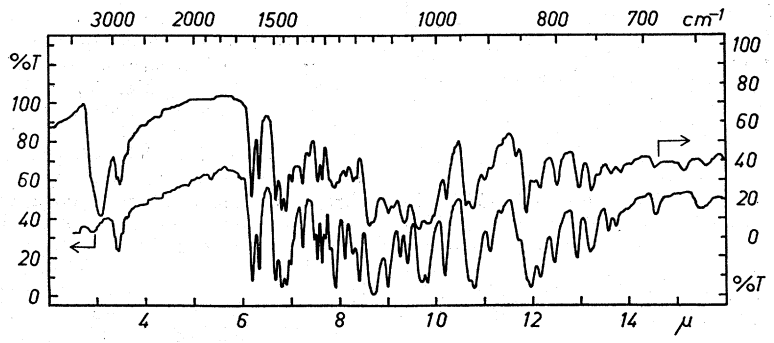


Fig. 2. Infrared spectra of trifolirhizin (above, right scale, 2.2 mg/300 mg KBr) and pterocarpin (below, left scale, 2.0 mg/300 mg KBr).

Experimental. For details of the isolation, see Hietala [1]. A good concentrate of the compound can be obtained by analogous operation in a series of about ten separatory funnels. Trifolirhizin crystallizes well from methanol, forming rods, m.p. 142–144° (decomp.), $[\alpha]_D^{20}$ −183° (ethanol, *c* 1.5). (Found: C 57.89; H 5.58; O 35.96; O–CH_3 5.51, 7.35; C–CH_3 0.00. Calc. for $C_{22}H_{22}O_{10} \cdot CH_3OH$: C 57.73; H 5.48; O 36.78; O–CH_3 6.5. Crystallization from benzene gave C 59.46; H 5.23; O 33.52; O–CH_3 0.32. Calc. for $C_{22}H_{22}O_{10}$: C 59.19; H 4.97; O 35.84.) The compound is difficultly soluble in benzene, easily soluble in warm methanol. It does not react with 2,4-dinitrophenylhydrazine under normal conditions. Hydrogenation with platinum in ethanol or acetic acid at room temperature gave unchanged starting material (mixed m.p., IR). *Acetate:* The compound was acetylated with acetic anhydride and pyridine (30 min, water bath) and crystallized from methanol, cluster of needles, m.p. 188–189°, $[\alpha]_D^{20}$ −126° (acetone, *c* 1). (Found: C 58.49, 58.23; H 4.94, 5.07; O 35.76, 36.52; O–CH_3 0.00; Acetyl 27.74 %. Calc. for $C_{30}H_{30}O_{14}$: C 58.63; H 4.92; O 36.45; Acetyl (4–OH) 28.0 %.) The infrared spectrum had no absorption in the hydroxyl stretching range. *Hydrolysis experiments:* The compound (51 mg) was dissolved in acetic acid (10 ml) and conc. HCl (1 ml) was added. The solution was kept at room temperature for 20 h and was then warmed on a water bath for 30 min. After addition of water the solution was extracted with ether. The extracted aqueous solution was poured through a column of IR-120 in H-form and the column washed with water. The eluate was repeatedly evaporated to a small volume and rediluted with water; finally it was evaporated to dryness. The residue was extracted with hot ethanol and the ethanol solution evaporated. Paper chromatography of the evaporation residue in three different solvent systems (phenol-NH_3, butanol-acetic acid-water, ethyl acetate-pyridine-water) gave spots with the same R_F values as glucose. The osazone, prepared in the usual way, melted at 202–204° and gave no depression with authentic material. Upon evaporation the ether solution gave a dark, semisolid mass (27 mg), with a maximum in the ultraviolet at 286 mμ. No crystalline material could be obtained from this residue. Alkaline hydrolysis of trifolirhizin with boiling 1 N NaOH in methanol for 1.5 h yielded, after acidification and extraction as above, upon paper chromatography of the water phase, a spot with the same R_F value as glucose (butanol-acetic acid-water). No crystalline material could be obtained from the ether phase.

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Table 2. Weight in grams of lipid fractions isolated from 100 g of muscle by chloroform-methanol extraction, followed by silicic acid chromatography.

	Beef	Pork
Total lipids	4.5699	6.6098
Non-phosphorus lipids	3.5539	5.8615
Fraction Ia (triglycerides)	2.8962	5.4473
Fraction Ib (triglycerides)	0.6577	0.4142
Phospholipids	1.0040	0.7462
Fraction II (cephalins)	0.4330	0.3155
Fraction III (lecithins and sphingomyelins)	0.4990	0.3737
Denatured proteins	0.0720	0.0570
Total recovery	4.5489 (99.5%)	6.6077 (99.9%)

by the formation of 2, 4-dinitrophenylhydrazones of long-chain carbonyl compounds after mild acid hydrolysis of these fractions, and to the formation of oxidized phospholipids as shown by the characteristic ultraviolet absorption at approx 235 mμ and 275 mμ (Lea, 1957).

The identities of the saturated acids and oleic, linoleic, linolenic, and arachidonic acids were established by direct comparison of retention volumes of the methyl esters with those of authentic specimens. The other assignments were based on plots of the log of retention volumes versus the number of carbon atoms. Saturated fatty acid methyl esters fall along a straight line—those containing one double bond along a parallel line, those with two double bonds along a second parallel line, etc. (Hornstein *et al.*, 1960a). As the number of double bonds in the molecule increases, the distance between these parallel lines tends to decrease; and it is possible, for example, that the "envelope" assigned to arachidonic acid may contain higher unsaturated C_{20} acids. The assignment made for tetradecenoic, tetradecadienoic, palmitoleic, eicosatrienoic, and docosadienoic should be considered rendered highly probable rather than absolute, since they were not based on direct comparison with authentic samples. Table 4 shows the fatty acid composition of the combined triglyceride fractions and of the combined phospholipid fractions. Unsaturated acids containing two or more double bonds make up 10% of the triglyceride fraction and about 50% of the phospholipid fraction; however, the actual milligrams of these acids contributed by the two fractions are similar since the ratio of triglycerides to phospholipids is about 4 : 1 in beef and about 8 : 1 in

Table 3. Fatty acid composition of lipid fractions (% of total fatty acids) obtained from lean beef and pork. Fractions Ia and Ib triglycerides, Fraction II cephalins, Fraction III lecithins and sphingomyelins.

	Fraction Ia		Fraction Ib		Fraction II		Fraction III	
Acid	Beef	Pork	Beef	Pork	Beef	Pork	Beef	Pork
Capric	....	....	1.1	2.7	....	....	....	....
Lauric	....	0.2	0.6	0.4	....	....	....	....
Myristic	2.1	1.1	2.8	2.0	1.4	3.6	3.6	1.0
Tetradecenoic	1.0	....	1.5	....	0.6	0.1	1.1	0.3
Tetradecadienoic	0.6	....	0.6	....	0.6	0.8	1.3	0.5
Palmitic	28.3	24.0	19.8	22.5	2.8	4.1	21.0	30.5
Palmitoleic	4.6	7.2	5.9	9.8	1.8	2.4	2.4	2.4
Stearic	17.0	12.1	16.2	7.0	27.4	20.6	6.6	4.6
Oleic	42.5	45.8	30.6	39.4	13.8	14.3	26.9	17.4
Linoleic	3.0	7.9	17.2	15.3	16.2	15.2	23.6	36.3
Linolenic	0.9	1.7	2.5	1.7	2.1	0.4	1.7	1.4
Eicosatrienoic	....	....	....	....	....	4.0	3.2	....
Arachidonic	....	....	1.2	1.2	33.3	32.4	8.6	5.6
Docosadienoic	....	....	....	....	....	2.1	....	....
Weight of fraction (g)	2.8962	5.4473	0.6577	0.4142	0.4330	0.3155	0.4900	0.3737

Table 4. Fatty acid composition of combined triglyceride fractions and of combined phospholipid fractions from Table 3 (% of total fatty acids).

Acid	Triglycerides		Phospholipids	
	Beef	Pork	Beef	Pork
Capric	0.1	0.1	....	....
Lauric	0.1	0.2	....	....
Myristic	2.2	1.2	2.6	2.0
Tetradecenoic	1.0	....	0.9	0.2
Tetradecadienoic	0.6	....	1.3	0.6
Palmitic	27.5	23.9	13.2	20.0
Palmitoleic	4.7	7.4	2.2	2.3
Stearic	16.9	11.6	15.6	11.0
Oleic	41.3	45.2	21.2	16.2
Linoleic	4.4	8.7	20.2	27.9
Linolenic	1.1	1.6	1.8	1.0
Eicosatrienoic	....	....	1.8	1.6
Arachidonic	0.1	0.1	19.2	16.3
Docosadienoic	....	....	....	0.9
Total saturated acids	46.7	37.0	31.4	33.0
Total monounsaturated acids	47.1	52.6	24.3	18.8
Total dienoic acids	5.0	8.7	21.5	29.3
Total trienoic acids	1.1	1.6	3.7	2.6
Total tetraenoic acids	0.1	0.1	19.1	16.3

pork. The major quantitative difference is the large amount of arachidonic acid (possible maximum 150 mg per 100 g of muscle in beef and 100 mg/100 g of muscle in pork) contributed by the phospholipid fraction that has no counterpart in the triglyceride fraction (Table 3).

Flavor characteristics. We attempted to evaluate the aromas developed by heating freshly isolated lipid fractions in air. The odors of the beef and pork triglyceride fractions were respectively reminiscent of "fried-fat" and "bacon." The cephalin fractions from both beef and pork produced strong "fishy" odors, probably attributable to the high arachidonic acid content of these fractions. The heated lecithin-sphingomyelin fractions from both beef and pork were also alike in odor, the "fishy" smell less pronounced than in the cephalin fraction and superimposed on an aroma suggestive of liver. Total lipid and phospholipid extracts of pork and beef were exposed to air, and their odors noted at 24-hr intervals. Rancid odors developed more quickly in these samples than in neutral fat. At the end of one week, the samples were all highly rancid. The odor of the pork total lipid extract was the least objectionable; the large amount of triglyceride present either dissolved the potent odor compounds produced, thus lowering their vapor pressure, or mechanically inhibited the oxidation of the phospholipids by limiting their surface exposed to air. It was concluded that phospholipids did not contribute to desirable meat flavor and that the possibility existed that in excessively lean meat they could contribute to poor flavor. The triglyceride contribution was similar to that previously described (Hornstein and Crowe, 1960). Changes in color were also noted in these fractions. The phospholipids darkened rapidly, the total lipid fraction changed color more slowly, and the neutral lipids showed the least color change.

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